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transfected transgene in both RNA and protein products.
In this fashion, other pairs of drug selection
expression vectors may be employed to produce cell
lines transfected with multiple vectors which have
5   stably integrated high copy numbers of the genetic
material and express various levels of biologically
active molecules of interest.

        A multiplicity of cells may be used in the
methods of this invention, such that implantation of a
10 polymer-capsule can be sufficient to provide an
effective amount of the needed substance or function to
an individual. In addition, more than one biologically
active molecule may be stably expressed and/or
delivered over long periods from a single capsule.
15   One way to accomplish this result is to
encapsulate a single cell line which has been
genetically altered to express more than one
heterologous gene.

        Another way to accomplish this result is to
20 encapsulate in a single capsule a mixture of cells,
wherein some cells have been genetically modified to
express one biologically active molecule and other
cells have been genetically modified to express a
second biologically active molecule. It will be
25 appreciated that a non-genetically engineered cell line
can be utilized to provide the second biologically
active molecule.

        For example, a cell line may also be
genetically engineered to express different
30 biologically active molecules. The sub clones of the
parental cell lines, each expressing a different
transgene, may then be pooled and encapsulated to
achieve the desired effect on a long-term basis.

        These approaches would eliminate the need for
35 using multiple implants for the long-term, stable

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expression and delivery of more than one biologically
active molecule to a single site.

This invention also contemplates using
capsules containing cells that are genetically modified
5   with a heterologous gene, which gene enables the cells
to remain viable within the capsule upon implantation
within a host mammal. In other words, the methods of
this invention are also directed to methods of delivery
of molecules within the implanted capsules.

10   In a specific embodiment of this invention we
used the pNUT expression vector containing the human
β-NGF gene operatively linked to the mouse
metallothionein promoter to transfect BHK cells via the
calcium phosphate co-precipitation method.

15   A variety of biocompatible immunoisolatory
capsules are suitable for delivery of molecules
according to this invention. Such capsules will allow
for the passage of metabolites, nutrients and
therapeutic substances while minimizing the detrimental
20   effects of the host immune system. Preferably the
capsule of this invention will be similar to those
described in Aebischer et al., PCT publication
WO 92/19195, incorporated herein by reference.

Most preferably the T1/2 membranes described
25   herein are used to encapsulate the cells that are
modified according to the methods of this invention.
It will be appreciated that the T1/2 membranes
described herein can also be used for encapsulation of
any other suitable cell or cell lines. Thus, the T1/2
30   membranes of this invention can be used to encapsulate
primary (non-dividing) cells, as well as dividing
cells.

Useful biocompatible polymer capsules
comprise (a) a core which contains a cell or cells,
35   either suspended in a liquid medium or immobilized

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within a hydrogel or extracellular matrix, and (b) a
surrounding or peripheral region of permselective
matrix or membrane (jacket) which does not contain
isolated cells, which is biocompatible, and which is
5   sufficient to protect isolated cells if present in the
core from detrimental immunological attack.

The core of the polymer capsule is
constructed to provide a suitable local environment for
the continued viability and function of the cells
10   isolated therein.

Many transformed cells or cell lines are most
advantageously isolated within a capsule having a
liquid core. For example, cells can be isolated within
a capsule whose core comprises a nutrient medium,
15   optionally containing a liquid source of additional
factors to sustain cell viability and function, such as
fetal bovine or equine serum.

Microcapsules may sometimes be suitable for
use in the methods and compositions of this invention.
20   The fabrication of microcapsules have been described in
Espevik et al., PCT publication WO 9107951, and Sefton,
United States patent 4,353,888 incorporated herein by
reference.

Suitably, the core may be composed of a
25   matrix formed by a hydrogel which stabilizes the
position of the cells in cell clumps. The term
"hydrogel" herein refers to a three dimensional network
of cross-linked hydrophilic polymers. The network is
in the form of a gel, substantially composed of water,
30   preferably but not limited to gels being greater than
90% water.

Compositions which form hydrogels fall into
three classes. The first class carries a net negative
charge (e.g., alginate). The second class carries a
35   net positive charge (e.g., collagen and laminin).

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Examples of commercially available extracellular matrix
components include Matrigel™ and Vitrogen™.
Fibroblasts generally survive well in a positively
charged matrix and are thus suitably enclosed in

5   extracellular-matrix type hydrogels. The third class
is net neutral in charge (e.g., highly crosslinked
polyethylene oxide, or polyvinylalcohol). Any suitable
matrix or spacer may be employed within the core,
including precipitated chitosan, synthetic polymers and

10 polymer blends, microcarriers and the like, depending
upon the growth characteristics of the cells to be
encapsulated.

           Preferably, the capsules are immunoisolatory.
To be immunoisolatory, the surrounding or peripheral

15 region of the capsule should confer protection of the
cells from the immune system of the host in whom the
capsule is implanted, by preventing harmful substances
of the host's body from entering the core of the
vehicle, and by providing a physical barrier sufficient

20 to prevent detrimental immunological contact between
the isolated cells and the host's immune system. The
thickness of this physical barrier can vary, but it
will always be sufficiently thick to prevent direct
contact between the cells and/or substances on either

25 side of the barrier. The thickness of this region
generally ranges between 5 and 200 microns; thicknesses
of 10 to 100 microns are preferred, and thickness of 20
to 75 microns are particularly preferred. Types of
immunological attack which can be prevented or

30 minimized by the use of the instant vehicle include
attack by macrophages, neutrophils, cellular immune
responses (e.g. natural killer cells and antibody-
dependent T cell-mediated cytoloysis (ADCC), and
humoral response (e.g., antibody-dependent complement

35 mediated cytolysis).

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Use of immunoisolatory capsules allows the
implantation of xenogeneic cells or tissue, without a
concomitant need to immunosuppress the recipient. Use
of immunoisolatory capsules also allows use of
5   unmatched cells (allografts). The type and vigor of an
immune response to xenogeneic cells is expected to
differ from the response encountered when syngeneic or
allogeneic tissue is implanted into a recipient. This
response may proceed primarily by cell-mediated, or by
10 complement-mediated attack; the determining parameters
in a particular case may be poorly understood.
However, the exclusion of IgG from the core of the
vehicle is not the touchstone of immunoprotection,
because in most cases IgG alone is insufficient to
15 produce cytolysis of the target cells or tissues.
Using immunoisolatory macrocapsules, it is possible to
deliver needed high molecular weight products or to
provide metabolic functions pertaining to high
molecular weight substances, provided that critical
20 substances necessary to the mediation of immunological
attack are excluded from the immunoisolatory capsule.
These substances may comprise the complement attack
complex component Clq, or they may comprise phagocytic
or cytotoxic cells; the instant immunoisolatory capsule
25 provides a protective barrier between these harmful
substances and the isolated cells. Thus, an
immunoisolatory capsule can be used for the delivery
even from xenogeneic cells, products having a wide
range of molecular sizes, such as insulin, parathyroid
30 hormone, interleukin 3, erythropoietin, albumin,
transferrin, enkephalins, endorphins, catecholamines,
Factor VIII, NGF, BDNF, NT-3, NT-4/5, CNTF, GDNF,
CDF/LIF, EGF, IGF, bFGF, aFGF, PDGF, TGF and the like.
Various polymers and polymer blends can be
35 used to manufacture the capsule jacket, including

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polyacrylates (including acrylic copolymers),
polyvinylidenes, polyvinyl chloride copolymers,
polyurethanes, polystyrenes, polyamides, cellulose
acetates, cellulose nitrates, polysulfones,
5 polyphosphazenes, polyacrylonitriles,
poly(acrylonitrile/covinyl chloride), as well as
derivatives, copolymers and mixtures thereof.

The capsule can be any configuration
appropriate for maintaining biological activity and
10 providing access for delivery of the product or
function, including for example, cylindrical,
rectangular, disk-shaped, patch-shaped, ovoid,
stellate, or spherical. Moreover, the capsule can be
coiled or wrapped into a mesh-like or nested structure.
15 If the capsule is to be retrieved after it is
implanted, configurations which tend to lead to
migration of the capsules from the site of
implantation, such as spherical capsules small enough
to travel in the recipient's blood vessels, are not
20 preferred. Certain shapes, such as rectangles,
patches, disks, cylinders, and flat sheets offer
greater structural integrity and are preferable where
retrieval is desired.

The instant capsule must provide, in at least
25 one dimension, sufficiently close proximity of any
isolated cells in the core to the surrounding tissues
of the recipient, including the recipient's
bloodstream, in order to maintain the viability and
function of the isolated cells. However, the
30 diffusional limitations of the materials used to form
the capsule do not in all cases solely prescribe its
configurational limits. Certain additives can be used
which alter or enhance the diffusional properties, or
nutrient or oxygen transport properties, of the basic
35 vehicle. For example, the internal medium can be

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supplemented with oxygen-saturated perfluorocarbons,
thus reducing the needs for immediate contact with
blood-borne oxygen. This will allow isolated cells to
remain viable while, for instance, a gradient of
5 angiotensin is released from the capsule into the
surrounding tissues, stimulating ingrowth of
capillaries. References and methods for use of
perfluorocarbons are given by Faithful, N.S. (1987)
Anaesthesia, 42, pp. 234-242 and NASA Tech Briefs MSC-
10 21480, U.S. Govt. Printing Office, Washington, D.C.
20402, incorporated herein by reference.

        In one preferred embodiment, the implantable
capsule is of a sufficient size and durability for
complete retrieval after implantation. Such
15 macrocapsules have a core of a preferable minimum
volume of about 1 to 10μl and depending upon use are
easily fabricated to have a volume in excess of 100 μl.

        The preferred capsule will have an inner
single ultrafiltration membrane with a permselective
20 pore-size permeability range of 60-98% BSA rejection
coefficient and 50-90% ovalbumin rejection coefficient.
The capsule may be in the form of a flat sheet sealed
at the periphery or of a hollow fiber sealed at the
ends as described in PCT application WO 92/19195. In a
25 flat sheet format the two walls will be separated by a
gap thickness of less than 1000 microns, preferably
less than 300 microns. Wall thickness should be
between about 25-200 microns, preferably between about
30-75 microns.

30   In a hollow fiber configuration, the fiber
will have an inside diameter of less than 1500 microns,
preferably less than 300-600 microns. In either
geometry, the hydraulic permeability will be in the
range of 1-100 mls/min/M^2/mmHg, preferably in the range
35 of 25 to 70 mls/min/M^2/mmHg. The glucose mass transfer

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coefficient of the capsule, defined, measured and
calculated as described by Dionne et al., ASAIO
Abstracts, p. 99 (1993), and Colton et al., The Kidney,
eds., Brenner BM and Rector FC, pp. 2425-89 (1981) will
5   be greater than 10^{-6} cm/sec, preferably greater than
10^{-4} cm/sec.

The morphology of the outer wall surface of
the capsule is variable. Previously described T1, T2,
T4 membranes and the novel T1/2 membranes of this
10 invention differ in their outer wall surface
morphology. All these membranes are characterized by
an inner permselective skin.

T1 membranes are characterized by an "open"
or fenestrated non-permselective outer surface wall,
15 and a trabecular wall structure between the outer and
inner wall surfaces. See, e.g. Lacy et al., Science,
254, pp. 1782-84 (1991). The fenestrations of
"macropores" in the outer wall surface of a T1 membrane
typically occupy about 20%-40% of the total outer
20 surface wall area. Typically, the macropores are 10μm-
15μm in diameter or greater.

T2 membranes have a similar trabecular
structure between the inner and outer walls but are
characterized by a more "closed" or smoother outer
25 surface wall. T2 membranes, typically are
characterized by fewer than 10% macropores on the outer
surface wall and virtually no macropores in the 5-15 μm
diameter size range.

T4 membranes are further distinct in that the
30 outer surface is also permselective, unlike the T1 or
T2 membranes. T4 membranes are useful for CSF
implantation sites, such as the ventricles or sub-
arachnoid space, as well as other fluid bathed
implantation sites.

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        For primate brain parenchyma, or other
"solid" tissue implantation sites, we prefer to employ
a smooth wall capsule which is substantially
impermeable to cells.   Others have described open walls
5 which encourage microvascularization (see, e.g.,
Brauker et al., WO/92/07525).   T1 and T1/2 capsules are
especially suited for long term implants.   We prefer
T1/2 capsules.
        T1/2 capsules are characterized by a total
10 macropore distribution of between approximately 2-20%,
preferably 2-15% of the total outer surface wall area.
The macropores should fall within the size range of
approximately 5 μm to about 15 μm in diameter.   The
relative distribution of pore sizes within this range
15 can vary.
        In one specific embodiment T1/2 hollow fiber
membrane capsules made from PAN/PVC were utilized.
According to this embodiment, the total macropore area
was about 12% of the total outer wall surface area.
20 Approximately 20% of the macropores ranged between 5-
10 μm in diameter and about 80% of the macropores were
about 10 μm in diameter.
        In another specific embodiment, T1/2 hollow
fiber membrane capsules were fabricated having a total
25 macropore area of about 2.4% of the total outer wall
surface area.   Approximately 17% of the macropores were
about 5 μm in diameter, about 33% were about 10 μm in
diameter, and about 50% were about 15 μm in diameter.
        In another specific embodiment, T1/2 hollow
30 fiber membrane capsules were fabricated having a total
macropore area of approximately 10% of the total outer
wall surface area.   Greater than 99% of these
macropores ranged between 10-15 μm in diameter.

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flow rates using well known techniques described by
Cabasso, I., Encyclopedia of Chemical Technology, 12,
pp. 492-517 (1980). The coagulant which can include
biological tissue fragments, organelles, or suspensions
5   of cells and/or other therapeutic agents, as described
in Dionne, WO 92/19195 and United States Patents
5,158,881, 5,283,187 and 5,284,761, incorporated herein
by reference.

          According to those methods, T1 membranes may
10   be formed by coextrusion of a polymer solution and
coagulant solution through air before entering a quench
bath. T2 membranes may be formed by coextruding the
polymer, and coagulation solutions into humidified air
or a mist and then into a bath. T4 membranes may be
15   formed by coextrusion of the polymer and coagulant
solutions directly into a coagulant bath, so that
formation of the permselective membrane occurs on both
outer and inner wall surfaces simultaneously.

          T1/2 membranes may be formed using similar
20   methods used to form T2 membranes. However, the mist
or humidity at the coextrusion port may be controlled
according to known methods to produced the desired
outer surface morphology. Alternatively, the nozzle
distance from a quench bath may be varied, according to
25   routine methods. Further, if coextrusion is used to
cast the membrane, the absolute and/or relative flow
rates of polymer and coagulant may be adjusted to
achieve the desired outer wall surface morphology.
Finally, the polymer and coagulant solution
30   compositions and temperatures can be varied to achieve
the desired outer surface wall morphology. For
example, the casting solution may be 10-15% PAN/PVC in
DMSO (w/w) and the coagulant may be water, or other
aqueous medium. Alternatively, the casting solution

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may be, e.g., 16% PAN/PVC, and the coagulant may be,
e.g, 40% NMP, 60% H_2O at 23°C.

Any suitable method of sealing the capsules
may be used, including the employment of polymer
5   adhesives and/or crimping, knotting and heat sealing.
These sealing techniques are known in the art. In
addition, any suitable "dry" sealing method can also be
used. In such methods, a substantially non-porous
fitting is provided through which the cell-containing
10   solution is introduced. Subsequent to filling, the
capsule is sealed. Such a method is described in
copending United States application Serial No.
08/082,407, herein incorporated by reference.

The methods and devices of this invention are
15   intended for use in a mammalian host, recipient,
subject or individual, preferably a primate, most
preferably a human.

A number of different implantation sites are
contemplated for the devices and methods of this
20   invention. These implantation sites include the
central nervous system, including the brain, spinal
cord, and aqueous and vitreous humors of the eye.
Preferred sites in the brain include the striatum, the
cerebral cortex, subthalamic nuclei and nucleus Basalis
25   of Maynert. Other preferred sites are the
cerebrospinal fluid, most preferably the subarachnoid
space and the lateral ventricles. This invention also
contemplates implantation into the kidney subcapsular
site, and intraperitoneal and subcutaneous sites, or
30   any other therapeutically beneficial site.

In an embodiment of this invention, methods
are provided for the treatment of diseases caused by
neural degeneration. Examples of human diseases which
are thought to be associated with neural degeneration
35   include Alzheimer's disease, Huntington's disease,

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AIDS-related dementia, Amyotrophic Lateral Sclerosis
(ALS) and Parkinson's disease.

Some animal models for neurodegenerative
conditions are based on the premise that a specific
5   insult may damage or kill neurons. In some cases this
may even lead to a cascade of neuronal death which
affects trophically interdependent neurons along
pathways responsible for specific brain functions.

A strategy for treatment of neural
10   degenerative conditions involves the localized
administration of growth or trophic factors in order to
(1) inhibit further damage to postsynaptic neurons, and
(2) improve viability of cells subjected to the insult.
Factors known to improve neuronal viability include
15   NGF, BDNF, NT-3, NT-4/5, CNTF, GDNF, CDF/LIF, bFGF,
aFGF, IGF, neurotensin, and Substance-P.

In one animal model for neurodegenerative
excitotoxicity, the glutamate analog, quinolinic acid,
is injected stereotaxically into the brain region known
20   as the striatum and/or basal ganglia to produce
neuropathology and symptoms analogous to those of
patients suffering from Huntington's disease. Both the
model and actual Huntington's disease are characterized
by damage to neurons necessary for aspects of motor
25   control. Furthermore, one of the early symptoms of
Huntington's disease is loss of body weight (Sanberg,
et al. Med J Aust., 1, pp. 407-409 (1981)). Similar
effects are also seen in the model system (Sanberg,
et al. Exp Neurol, 66, pp. 444-466 (1979)). Quinolinic
30   acid is also found at abnormally high levels in humans
suffering from AIDS-related dementia.

Huntington's disease (HD) is an autosomal
dominant disorder characterized by a progressive
dementia coupled with bizarre uncontrollable movements
35   and abnormal postures. HD is found in nearly all

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ethnic and racial groups with the prevalence rate in
the U.S. approximately 50/1,000,000 (Emerich, D.F. &
Sanberg, Neuromethods, 21, pp. 65-134 (1992)). The
manifestation of the disorder typically occurs in
5   middle life, about 35-45 years of age, followed by an
intractable course of mental deterioration and
progressive motor abnormalities with death usually
occurring within 15 years. Research into the neural
pathology in HD has revealed a complex mosaic of
10 related and interdependent neurochemical and
histopathological alterations.

A variety of avenues have been explored to
develop an animal model of HD. Recent investigations
have centered on the relationship between striatal
15 damage and the locomotor abnormalities resulting from
the use of selective cytotoxic compounds. Glutamate is
one of the major excitatory neurotransmitters found in
the CNS. It can act, however, as a potent neurotoxin
and a number of attempts have been made to develop
20 animal models of HD based on the relatively specific
cytotoxic effects of glutamate and other excitotoxic
compounds. These compounds include structural analogs
of glutamate, such as kainic acid (KA), ibotenic acid
(IA), and the endogenous tryptophan metabolite
25 quinolinic acid (QA). When injected into the brains of
rats, in extremely small doses, these compounds produce
a marked and locally restricted toxic effect while
sparing axons of passage and afferent nerve terminals.
The behavioral, neurochemical, and anatomical
30 consequences of excitotoxicity resemble those observed
in HD and have led to the speculation that an aberrant
overproduction or breakdown of endogenous excitotoxic
compounds is an etiological factor in HD.

Quinolinic acid, 2,3-pyridine dicarboxylic
35 acid, a metabolite of tryptophan, has attracted a great

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deal of attention because of its powerful excitotoxic
properties and wide distribution in both rat and human
brain (Schwarcz and Kohler, Neurosci. Lett., 38,
pp. 85-90 (1983); and Schwarcz et al., Science, 219,
5   pp. 316-318 (1983)). High concentrations of its
catabolic enzyme, quinolinic acid
phosphoribosyltransferase (QPRT), and immediate
anabolic enzyme, 3-hydroxyanthranilic acid (3HAO), have
been detected within the caudate suggesting that it
10   normally serves a role in striatal functioning (Foster
et al., Brain Res., 336, pp. 207-214 (1985)). The
striatum is among the structures most vulnerable to the
excitotoxic effects of QA (Schwarcz and Kohler, (1983),
supra) and neonatal, but not mature, animals appear to
15   be resistant to the toxic effects of QA corresponding
roughly to the typical onset of HD in middle age.
        Quinolinic acid has been reported to exert a
more selective degenerative effect in the striatum than
KA, which more closely resembles the pathology of HD
20   (Beal, Synapse, 3, pp. 38-47 (1989); and Roberts and
DiFiglia, Synapse, 3, pp. 363-371 (1989)). Like KA, QA
injections cause depletions of GABAergic neurons while
relatively sparing cholinergic neurons and axons of
extrinsic origin. Unlike KA or IA, intrastriatal
25   injections of QA appear to spare somatostatin- and
neuropeptide Y-containing neurons suggesting that this
model most closely reproduces the neuropathology
observed in the disease (Beal et al., J. Neuro Sci, 8,
pp. 3901-3908 (1988).
30   According to the present invention, trophic
factors are provided to the proper brain region by
implanting a capsule containing cells, including
genetically altered cells which secrete an appropriate
factor. In some instances, the genetically altered

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cells are autologous to the host and may not require
encapsulation.

Nerve growth factor-secreting cells such as
BHK cells engineered to express human NGF represent a
5 therapy for quinolinic acid induced neurodegeneration.

Another animal model involves lesion of the
fimbria-fornix (rodents) or fornix (primates). In
particular, neurons of the septohippocampal system are
axotomized which leads to NGF-dependent degeneration
10 and cell death in the septal cholinergic neurons.
These lesions cause degenerative changes in brain areas
similarly affected in Alzheimer's disease in humans.

According to the methods of this invention,
NGF may be delivered to the affected area by the
15 implantation of a capsule containing genetically
altered cells which secrete NGF. Other neurotrophic
factors such as CNTF, BDNF, bFGF, CDF/LIF may also
protect similar or non-overlapping populations of
septal cholinergic neurons from atrophy and/or death.
20 Preferably, the cells are fibroblasts which have been
genetically engineered to produce recombinant human
NGF.

Fornix lesions also cause behavioral deficits
in the animal subjects of the model, most easily
25 observed in tasks involving learning and memory. It
has been reported that chronic administration of NGF to
rats with fimbria-fornix lesions accelerates the
animals' behavioral recovery (Wills et al. Behav. Brain
Res., 17, pp. 17-24 (1985)). In the present invention,
30 implantation of the polymer capsule containing NGF-
secreting cells provides a practical way to deliver NGF
continuously to the appropriate brain region of the
lesioned animal. The capsules of the present invention
offer a practical form of therapy and/or prophylactic
35 treatment for Alzheimer's victims whose conditions may

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be ameliorated by continuous delivery of NGF to
specific brain regions.

The methods and compositions of this
invention may be used for the treatment of age-related
5 cognitive defects resulting from neural degeneration.
Such treatment may augment cognitive performance, thus
providing a symptomatic benefit. Alternatively,
treatment may provide a neuroprotective effect,
although not a symptomatic benefit. Age-related
10 cognitive dysfunction and dementia in humans has been
related to neuronal degeneration, especially of
cholinergic basal forebrain neurons, and the decline of
cortical and hippocampal levels of ChAT (Coyle et al.,
Science, 219, pp. 1184-90 (1983); Whitehouse et al.,
15 Science, 215, pp. 1237-39 (1982); Phelps et al.,
Neurobiol. Aging, 10, pp. 205-07 (1989); Gage et al.,
Neurobiol. Aging, 9, pp. 645-55 (1988)). Preclinical
research attempting to produce a rodent model of
dementia by selectively destroying cholinergic basal
20 forebrain neurons has failed to produce large, lasting
deficits in cognitive function. Aged rats exhibit
neuropathology similar to that reported in aged and
demented patients. The spatial-learning Morris water
maze is extremely sensitive to the deleterious effects
25 of these pathological processes (Morris, J. Neurosci.
Meth., 11, pp. 47-60 (1984); Morris, Learning and
Motivation, 12, pp. 239-60 (1981)). This task has been
validated as a measure of age-related cognitive
function -- the performance of aged rats in this task
30 is not strongly related to their motor, sensorimotor or
visual deficits, factors which confound other tests of
learning and memory (Gamzu, Ann. NY Acad. Sci., 444,
pp. 370-93 (1985)).

As a cognitive task which requires the
35 development of a spatial map (Eichenbaum et al., J.

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<u>Neurosci.</u>, 10, pp. 3531-42 (1990)), the Morris water
maze seems analogous to nonverbal tests of cognitive
function that are especially sensitive to senescence
and dementing disorders in the clinical setting.
5   Therefore, this task seems to be valid for the
assessment of potential new treatments for dementia.
For example, several studies have reported that
exogenous NGF improves Morris water maze performance in
aged and learning-impaired rats. See, e.g., Fischer
10   et al., <u>Nature</u>, 329, pp. 65-68 (1987); Fischer et al.,
<u>J. Neurosci.</u>, 11, pp. 1889-1906 (1991).
            Because NGF does not readily cross the blood
brain barrier, its administration into the CNS requires
the use of invasive procedures which compromise the
15   integrity of the blood brain barrier. For example, in
the rodent preclinical studies that demonstrated the
potential efficacy of exogenous NGF, the NGF was
administered with osmotic minipumps or through chronic
intraventricular cannulae. Those techniques require
20   repeated infusions into the brain, either through
injections via the cannulae, or from pumps which must
be replaced every time the reservoir is depleted.
Every occasion in which the pump reservoir must be
replaced or the injection syringe reinserted through
25   the annulae represents another opportunity that
contaminants might be introduced into the brain, which
is especially susceptible to infection.
            Even with the careful use of sterile
procedures, there is risk of infection. It has been
30   reported that even in intensive care units,
intracerebroventricular catheters used to monitor
intracranial pressure become infected with bacteria
after about three days (Saffran, <u>Perspectives in
Biology and Medicine</u>, 35, pp. 471-86 (1992). In
35   addition to the risk of infection, there seems to be

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some risk associated with the infusion procedure.
Infusions into the ventricles have been reported to
produce hydrocephalus (Saffran et al., Brain Research,
492, pp. 245-254 (1989)) and continuous infusions of
5   solutions into the parenchyma is associated with
necrosis.

Use of fetal tissue is clouded by ethical
concerns and unencapsulated non-fetal cells may be
rejected or produce tumors. In addition, tissue taken
10 from fetal sources may be highly variable. By
encapsulating NGF-producing cells, exogenous NGF can be
supplied with a relatively low risk of infection,
without the use of fetal tissue, and without the risk
of tissue rejection or tumor development.

15   Finally, concerns have also been expressed
about whether exogenous NGF at the doses previously
used itself might prove harmful or toxic, perhaps even
accelerating the neurodegenerative processes associated
with Alzheimer's disease (Saffran, Perspectives in
20 Biology and Medicine, 35, pp. 471-86 (1992). It has
been suggested that exogenous NGF might accelerate
tangle formation, initiate axon sprouting of
perivascular sympathetic axons potentially leading to
changes in cerebral blood flow, or remodel the
25 projections of basal forebrain neurons in response to
the exogenous NGF such that not-yet-affected basal
forebrain neurons become dysfunctional and thus
accelerating the dementing process (Saffran,
Perspectives in Biology and Medicine, 35, pp. 471-86
30 (1992)).

According to one aspect of this invention,
the beneficial effects of exogenous NGF for the
treatment of age related cognitive defects, including
Huntington's disease, Parkinson's disease, Alzheimer's
35 and ALS, may be obtained with doses much lower than

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previously reported to be effective for exogenous hNGF
delivery.

Administration of the previously reported
doses of NGF may have undesirable side effects
5 including severe weight loss, pain, listlessness,
hypophagia and recurrence of herpes infection.

According to this invention, capsular
delivery of NGF, synthesized in vivo, to the brain
ventricles, brain parenchyma, or other suitable CNS
10 location, ranging from 1-1500 ng/day is desirable. The
actual dosage of NGF, or other suitable factor, can be
varied by implanting a fewer or greater number of
capsules. We contemplate delivery of 1-1500,
preferably 10-600, most preferably 50-500, ng
15 NGF/human/day, for ventricular delivery and 1-1500,
preferably 10-150, ng NGF/human/day for parenchymal
delivery. These dosage ranges are significantly lower
than those previously reported doses of NGF needed for
CBF neuronal sparing/sprouting in rodent studies and in
20 primate studies (17-350 μg/day), especially if the
dosages are normalized to account for brain volume
differences between rodents, primates and humans.
Tuzynski et al., J. Neurosci., 10, pp. 3604-14 (1990);
Koliatsos et al., Ann. Neurol., 30, pp. 831-840 (1991),
25 Koliatsos et al., Experimental Neurol., 112, pp. 161-
73 (1991); Dekker et al., Neuroscience, 60, pp. 299-
309 (1994). In the one clinical patient evaluated, the
dose of NGF delivered was 75 μg/day. (Olson et al., J.
Neural Trans., 4, pp. 79-95 (1992)). In one
30 embodiment, genetically-modified cells secreting human
NGF (hNGF) are encapsulated in semipermeable membranes,
and implanted intraventricularly or intraparenchymally
in a suitable mammalian host, preferably a primate,
most preferably a human.

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In order that this invention may be better
understood, the following examples are set forth.
These examples are for purposes of illustration only,
and are not to be construed as limiting the scope of
5   this invention in any manner.

Examples

EXAMPLE 1:   Encapsulated NGF-Secreting BHK Cells
              Implanted In The CNS of Adult Rats

Human β-NGF expression and BHK cell line production

10   The human gene for β-NGF coding for the
complete amino acid sequence of the pre-pro form of NGF
was subcloned behind the mouse metallothionein promoter
in an expression construct that contains the mutant
form of dihydrofolate reductase (see, e.g., Kaufman
15   United States patent 4,470,461) driven by the SV40
promoter (Fig. 1).
            Two human genomic clones coding for the 5'
and 3' ends of the β-NGF gene were purchased from ATCC
(phbeta N8D8, phbeta N8B9). A 440 bp 5' Sca1-EcoR1
20   fragment from phbeta N8D8 was ligated to a 3' 2.0 kb
EcoR1 fragment isolated from phbeta N8B9. The spliced
NGF genomic sequence contained ~37 bp of the 3' end of
the first intron, the double ATG sequence believed to
be the protein translation start for pre-pro-NGF and
25   the complete coding sequence and entire 3' untranslated
region of the human gene (Hoyle et al., Neuron 10,
pp. 1019-1034 (1993)). The combined 2.51 kb β-NGF
construct was subcloned into the DHFR based pNUT
expression vector (Baetge et al., Proc. Natl. Acad.
30   Sci. USA, 83, pp. 5454-5458 (1986)) immediately
downstream from the mouse metallothionein-I promoter
(-650 to +7) and the first intron of the rat insulin II
gene (Palmiter R.D. et al., Proc. Natl. Acad. Sci. USA,
88, pp. 478-482 (1991)). The pNUT-β-NGF construct (see

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Fig. 1) was introduced into BHK cells using a standard
calcium phosphate-mediated transfection method.
Transfected BHK cells were selected in medium
containing 200 μM methotrexate (Sigma) for 3-4 weeks
5   and resistant cells were maintained as a polyclonal
population either with or without 200 μM methotrexate.

Quantitation of NGF bioactivity

Nerve growth factor (NGF) causes a marked
outgrowth of neurite processes in PC12 cells and as
10   such provides a rapid and sensitive assay for NGF
bioactivity. To test for the bioactivity of the NGF
produced by the NGF-transfected BHK cells, conditioned
medium (CM) from parental BHK cells (BHK-control) and
BHK-NGF cells were added to the PC12A (Schweitzer and
15   Kelly, J. Cell Biol., 101, pp. 667-676 (1985)) cells
grown on 6 well standard tissue culture plates. As a
control, 25 S mouse NGF was added to some of the wells
to induce neurites (50 ng/ml). The PC12A cells were
scored for neurites that were ≥ 3 times the length of
20   the cell body diameter over a period of 1-4 days. NGF
bioassays were also performed upon retrieval of
implanted control and NGF-secreting, BHK cell-loaded,
capsules by adding CM from the capsules to naive PC12A
cells taken from capsules incubated with fresh medium
25   for 24 hours. In all of the experiments, CM from BHK-
NGF cells produced a robust neurite outgrowth in PC12A
cells within 24 hours indicating that the NGF produced
from the BHK cells was bioactive. BHK-controls showed
no such capacity to elicit neurite outgrowth in the
30   PC12A cells in parallel experiments.

To determine whether the neurite outgrowth
was due to factors other than NGF, such as bFGF, we
added an NGF blocking antibody (mouse anti-β-NGF;
Boehringer-Mannheim Cat. # 1008-220) in combination

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with CM and recombinant hNGF. The addition of this
blocking antibody fully inhibited neurite outgrowth
from the PC12A cells in a dose-dependent fashion.
Finally, we performed a set of experiments probing the
5   BHK-control cell conditioned medium by ELISA for the
presence of basic fibroblast growth factor (bFGF).
Using a bFGF ELISA (Research and Diagnostics Systems,
Quantikine™, human FGF basic ELISA kit; Burgess et al.
Ann. Rev. Biochem, 58, p. 575 (1989)), we were unable
10 to detect any bFGF in heavily conditioned medium from
the BHK cells. Both of these experiments clearly
indicate that the neurite outgrowth seen in the PC12A
cells in response to BHK-NGF CM is due to the presence
of secreted NGF.

15 **NGF ELISA**

The quantitation of NGF expression from the
encapsulated and the unencapsulated BHK-NGF cells was
performed as follows: All of the reagents were
obtained from Boehringer Mannheim Biochemicals unless
20 otherwise noted. Nunc-Immuno MaxiSorp ELISA plates
were coated with 150 μl per well of anti-mouse-β(2.5S)
nerve growth factor at 1 ng/ml in coating buffer (1xPBS
without $CACl_2$ and without $MgCl_2$/0.1% sodium azide; pH
9.6). The coated plates were incubated at 37°C for at
25 least 2 hours or alternatively at 4°C overnight. The
coating solution was withdrawn from the wells and the
wells were washed three times with 300 μl wash buffer
(50 mM Tris-HCl/200 mM NaCl/10 mM $CaCl_2$/1% Triton
X-100/0.1% sodium azide; pH 7.0). The wells were then
30 blocked with 300 μl of coating solution with 10 mg/ml
of bovine serum albumin (BSA) at room temperature for
at least 30 minutes. The wells were then washed three
times with 300 μl wash buffer. Conditioned medium
samples were diluted 1:1 in 2x sample buffer (the

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sample buffer is the same as wash buffer, only with 2%
BSA). 100 µl of the prepared samples were loaded into
the wells. The plates were covered and then incubated
for at least 2 hours at 37°C or overnight at 4°C. The
5 solutions were removed from the wells by suction and
washed three times with 300 µl of wash buffer. To each
well, 100 µl of 4U/ml of anti-mouse-ß (2.5S) nerve
growth factor-ß-gal conjugate was added. The plates
were incubated at 37°C for at least 1 hour. The
10 solutions were removed from the wells by suction and
washed three times with 300 µl of wash buffer.
Finally, 200 µl of chlorophenol red-ß-D-
galactopyranoside substrate solution (40 mg CPRG in
100mM Hepes/150 mM NaCl/2 mM $MgCl_2$/0.1% sodium azide/1%
15 BSA; pH 7.0) was added to the wells and incubated at
37°C. After approximately 30 minutes to one hour or
after the color development was sufficient for
photometric determination at 570 nm, the samples were
analyzed on a plate reader and measured against
20 recombinant NGF protein standards.

c-fos induction assay

Qualitative c-fos induction elicited by NGF
administration to PC12A cells was measured by an
immunofluorescence assay. PC12A cells were plated at a
25 density of 100,000 cells per ml on poly ornithine-
treated glass coverslips (12 mm) and allowed to
equilibrate for at least 24 hours in a 24-well plate.
Cells were grown in the same medium as previously
described in the section on neurite outgrowth bioassay.
30   To test for c-fos induction in the PC12A
cells, conditioned medium from capsules containing BHK
and BHK-NGF cells or recombinant human NGF (50 ng/ml)
was added to the PC12A cells for 2 hours and allowed to
incubate at 37ºC and 5% CO_2. Following this

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incubation, the coverslips were fixed with 4%
paraformaldehyde (in 0.1 M PBS, pH 7.4), washed 2 X
with 10mM glycine in PBS, permeabilized with 1% triton
X100 (in PBS for 10 minutes) and 1% nonidet P40 (in PBS
5   for 10 minutes). The cells on coverslips were washed
3 X 5 minutes with PBS, blocked with 5% normal goat
serum (NGS) in PBS for 1 hour and incubated in a rabbit
polyclonal antiserum (Oncogene Science) raised against
c-fos diluted 1:10 in 1% NGS in PBS for 3 hours. The
10 coverslips were then washed 2 X 5 minutes with PBS and
incubated with a fluorescein-conjugated goat anti-
rabbit IgG antibody. Finally, the coverslips were
washed 2X with PBS and mounted with Citifluor®
antifadent and viewed by fluorescence microscopy.
15 Fluorescence was monitored by microscopy and c-fos
induction measured by the presence of fluorescently
labeled nuclei.

Encapsulation procedure

        Asymmetric single skin hollow fibers were
20 cast from solutions of 12.5% poly (acrylonitrile vinyl
chloride, i.e. PAN-PVC) copolymer in dimethyl sulfoxide
(w/w). The fabrication process is known as phase
inversion using a dry-wet (jet) spinning technique
according to Cabasso, Encyclopedia of Chemical
25 Technology, 12, pp. 492-517 (1980). After the spinning
process, the hollow fibers were sterilely transferred
into a distilled water bath containing 25% glycerol,
which provides a method for keeping the pores intact
during the drying procedure (Cabasso, 1980, supra).
30 The fibers produced (XP 11) were a T1/2 membrane type,
having an inner diameter of 450 ± 25 μm, a hydraulic
permeability of 53 ml/(m^2 min mmHg), a BSA rejection
coefficient of 88.7 ± 2.1%, an ovalbumin rejection
coefficient of 82.0 ± 1.7%, and a glucose mass transfer

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coefficient of about 8×10^{-4} cm/s. After drying, devices
were fabricated by mounting a length of 6-7 mm dry
hollow fiber, with a distal seal, onto a light-cured
acrylate hub with a septal fixture at the proximal end
5   which has loading access for cells to be injected into
the lumen of the device. Glycerol was removed from the
devices with 70% filter sterilized ethanol and placed
in HBSS prior to the encapsulation procedure.

          Cells were loaded into the prefabricated
10   encapsulation devices as follows: either BHK-control
cells or BHK-NGF cells were loaded into prefabricated
devices at a density of approximately 10^{7}/ml. The BHK
cell suspensions at a density of 2×10^{7}/ml were mixed
1:1 with physiologic Vitrogen® (Celtrix, Palo Alto,
15   CA), and infused into the pre-fabricated devices
through the septal access port. After infusing 2-2.5
μl of the cellular suspension, the septum was cracked
off and the access port was sealed using a light-cured
acrylate (Zeneca). BHK cell-loaded devices were
20   maintained in a serum-free defined medium, PC1 (Hycor),
for 4-5 days prior to implantation. After 3 or 4 days
in vitro, the cell-loaded capsules were washed twice in
HBSS, and placed in 1 ml of fresh medium to be analyzed
for NGF by ELISA.

25   **Long Term ß-NGF Expression in Adult Rat CNS**

        Our in vitro experiments demonstrated long-
term, stable, high level expression of human ß-NGF in
BHK cells. To determine if this long-term, stable
expression could be achieved in vivo, we implanted
30   capsules containing BHK cells into the CNS of adult
Lewis rats.

        Prior to implantation, conditioned medium
(CM) taken from BHK-NGF and BHK-control capsules were
subjected to neurite outgrowth assay for NGF as

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described above. 0.5 ml of CM from the BHK-NGF cells
was equivalent to 50 ng/ml of exogenously added NGF, in
terms of the extens of neurite outgrowth, whereas CM
from the BHK-control cells did not contain detectable
5   levels of NGF.

The capsules were implanted into the striatum
of adult Lewis rats for one, three and six month
periods. Upon explanation, the capsules were tested
for NGF production by the neurite outgrowth assay.

10   After 1, 3, and 6 months in vivo, the BHK-
NGF loaded capsules were able to produce neurite
outgrowth in PC12A cells equivalent to or greater than
50 ng/ml of NGF. No NGF activity was detectable in the
CM from the BHK-control capsules. As shown in Table I,
15   ELISA quantitation of the samples from the encapsulated
BHK-NGF cells release up to about 20 ng/24 hr/capsule
after 3 and 6 months in vivo.

Table I

NGF AS MEASURED BY ELISA IN ng/CAPSULE/24 HR

Condition	Pre-	Post-
3 mo. nv1 (BHK-NGF)	NA	17.1
3 mo. nv2 (BHK-NGF)	NA	13.1
3 mo. nv3 (BHK-CONTROL)	NA	0.1
6 mo. nv1 (BHK-NGF)	NA	21.6
6 mo. nv2 (BHK-NGF)	NA	2.8
6 mo. nv3 (BHK-CONTROL)	NA	0.1

NA = Not Available; no medium samples for NGF ELISA
pre-implant

30   nv1, nv2 and nv3 represent naive animals

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EXAMPLE 2:   Fimbria-Fornix Lesion Study in Rats

         To evaluate the ability of encapsulated NGF-
secreting BHK cells (as described in Example 1) to
release efficacious amounts of NGF in vivo, fimbria-
5   fornix aspirative lesions were stereotaxically
performed in 14 Lewis rats. Immediately after
lesioning, a BHK-control or BHK-NGF loaded XP-11
device, as described in Example 1, was stereotaxically
implanted into the lesion site.

10 **Subjects**

         Adult male Lewis rats (Harlan Breeders,
Indianapolis, IN) approximately 3 months old and
weighing approximately 300 grams were used in the
following studies. The animals were housed in groups
15 of three in a temperature and humidity controlled
colony room which was maintained on a 12 hour
light/dark cycle with lights on at 0700 hours.

Stereotaxic surgery

         Immediately prior to surgery, rats were
20 anesthetized with an intramuscular injection of a
ketamine, xylazine and acepromazine mixture and
positioned in a Kopf stereotoxic instrument (see
Emerich et al. 1992). A sagittal incision was made in
the scalp and a craniotomy performed extending 2.0 mm
25 posterior and 3.0 mm lateral from Bregma. An
aspirative device with a 20 gauge tip was mounted to a
stereotaxic frame (Kopf Instruments) and the medial
parietal cortex, cingulate cortex, corpus callosum,
dorsal hippocampus, dorsal thalamus and fimbria-fornix
30 were aspirated by placing the suction tip 1.40 mm
posterior to Bregma and lowering it immediately lateral
to the sagittal sinus to a depth of 5.0 mm. The tip
was then moved laterally in 0.5 mm increments until a

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position of 3.0 mm lateral to Bregma was attained.
Immediately following the aspiration, the rats were
unilaterally implanted with either transfected (N = 8)
or non-transfected BHK cell-containing capsules (N = 6)
5   by placing the capsule within an 18 gauge Teflon
catheter mounted to the stereotaxic frame. Each device
measured 0.7 cm in length by 600 microns in diameter
and contained approximately 15-25 x 10^3 cells.

        A stainless steel obdurator was placed within
10   the cannula, and the obdurator held in place while the
outer cannula was raised to passively place the capsule
within the previously prepared cavity. The stereotaxic
coordinates for implantation were: 0.5 mm posterior to
Bregma, 1.0 mm lateral to the sagittal suture and 7.5
15   mm below the cortical surface.

Histology

        Animals were anesthetized 3 weeks following
surgery and prepared for histological analysis.
Animals were transcardially perfused, using a
20   peristaltic pump, with the following: 20 ml saline
(0.9%, room temperature), 120 ml of glutaraldehyde
(0.1%), 500 ml 0.1% glutaraldehyde/4% paraformaldehyde,
300 ml of paraformaldehyde (4%), and finally 300 ml of
10% sucrose. All solutions were ice cold (4°C) and
25   prepared in phosphate buffered saline (pH = 7.4) unless
otherwise noted.

        Brains were removed following fixation,
placed in 25% buffered sucrose (pH = 7.4) and
refrigerated for 24-48 hours. Tissue was cut at 20 μm
30   intervals on a cryostat and mounted onto polylysine
coated slides. Every 3rd section throughout the septum
was saved and processed for choline acetyltransferase
(ChAT) immunoreactivity according to the following
protocol: (1) overnight incubation in PBS containing

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0.8% Triton X-100 + 10% normal serum, (2) 48 hour
incubation with primary antibody (goat antiserum to
ChAT; Chemicon) at a dilution of 1:1000, (3) 6 x 5
minute rinses in PBS + 0.2% Triton X-100 followed by a
5   1.5 hour incubation in biotinylated secondary antibody
(IgG), (4) 6 x 5 minute rinses in PBS + 0.2% Triton X-
100, (5) incubation with Avidin-Biotin Complex (ABC,
Vector elite) for 1.5 hours, (6) 3 x 5 minutes rinses
in PBS, (7) 5 minute rinse in distilled water,
10 (8) incubation with 3,3-diaminobenzidine (DAB) (0.05%)
+ 2% nickel ammonium sulfate dissolved in 0.1% Tris
buffer for 5 minutes followed by hydrogen peroxide
(0.01%) for 5 minutes, (9) the reaction was terminated
by 3 x 1 minute rinses in PBS.
15   Sections were mounted, dehydrated and
coverslipped. Adjacent sections were stained for
hematoxylin and eosin (H+E). To verify the extent of
lesion produced following aspirations of the fimbria-
fornix, every 10th section was taken throughout the
20 hippocampus and stained for acetylcholinesterase
according to the method of Van Ootegan et al. (Brain
Res. Bull., 12, pp. 543-553 (1984)). For
quantification of cholinergic cell loss, ChAT-positive
neurons were counted in the medial septum and vertical
25 limb of the diagonal band at a magnification of 10X.
Representative sections (3 per brain) located
approximately 0.7, 0.5 and 0.2 mm anterior to Bregma
from each animal were used for this analysis.

NGF Release Results

30   At 3½ weeks post implantation the animals
were anesthetized and the devices retrieved by gently
pulling the silicone tethers. Each capsule was
incubated for 24 hours in 1 ml of medium and CM was
assayed for NGF by ELISA. Pre- and post-

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transplantation NGF secretion levels are listed in
Table II.

Table II

NGF AS MEASURED BY ELISA IN ng/CAPSULE/24 HR

Condition	Pre-	± SD	Post-	± SD
F/F UNILAT (n=4)	27.2	2.5	5.2	0.9
CONTROLS (n=6)	0.1	0.05	0.15	0.1

Histological sections taken through the
hippocampus and septum of all animals were examined by
immunocytochemistry for acetylcholinesterase (AchE) in
the hippocampus and choline acetyltransferase (ChAT) in
the septum. AchE immunoreactivity in the hippocampus
was used as a second indicator of lesion completeness
and ChAT-immunoreactivity in the septum provided
evidence for cholinergic cell body sparing or atrophy
as a result of transplant and lesions. A
representative comparison of AchE immunostaining in the
hippocampus of the lesioned-vs-control side
demonstrated nearly complete loss of AchE afferents to
the lesioned side.

Total ChAT-positive neurons remaining in the
septum 3½ weeks post-lesion in BHK-control and BHK-NGF
implanted animals were counted and the combined results
are shown in Fig. 2. Quantitation of ChAT-positive
neurons revealed that with BHK-control capsules, only
15±3% of the neurons remained positive on the lesioned
side of the brain compared with the non-lesioned side,
whereas with the BHK-NGF capsules, 90±5% of the
cholineric neurons were immunopositive for ChAT.
Encapsulated cell survival was equivalent between the
BHK-control and BHK-hNGF cell-loaded capsules (data not
shown).

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EXAMPLE 3:   Primate Fornix Lesion

Example 2 demonstrated that the chronic delivery of hNGF from the BHK cells of this invention into the lateral ventricle of adult rats with fimbria-fornix lesions protects the medial septal cholinergic neurons that otherwise would have died as a result of the lesion.   Similar experiments are described here performed in non-human primates.   Lesioning of the fornix in monkeys (Cebus apella) is described in (Kordower and Fiandaca, J. Comp. Neurol., 298, pp. 443, (1990)).

Adult cynomolgous monkeys were used in these lesion experiments.   After the unilateral lesion was complete, 5 XP-11 capsule devices, as described in Example 1, (identified by number) were manually placed within the lateral ventricle adjacent to the medial septum, ipsilateral to the lesion.   As controls, some animals were implanted with devices loaded with BHK-control cells.   Following the implantation procedure, the surgical sites were closed and the animal were allowed to recover and were closely monitored for 3½ weeks.   Following the 3½ week survival period, the numbered capsules were carefully removed and placed in standard cell culture medium and then reassayed by ELISA for NGF release as they were pre-implantation (Table III).   The animals were then sacrificed by perfusing with aldehyde fixatives and the brains were sectioned and processed for histochemical and immunohistochemical procedures.

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : **C12N 5/06, 5/08**	**A1**	(11) International Publication Number: **WO 95/00632**
		(43) International Publication Date: 5 January 1995 (05.01.95)

(21) International Application Number: PCT/US94/06893

(22) International Filing Date: 17 June 1994 (17.06.94)

(30) Priority Data:
08/079,719   18 June 1993 (18.06.93)   US

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(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LV, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: MEDIUM FOR LONG-TERM PROLIFERATION AND DEVELOPMENT OF CELLS

(57) Abstract

A serum-free or serum-depleted medium for the short- and long-term proliferation and development of cells, particularly hematopoietic cells and bone marrow stromal cells, the medium comprising cell proliferation and development effective amounts of: a standard culture medium such as Iscove's modified Dulbecco's medium; serum albumin; transferrin; a source of lipids and fatty acids; cholesterol; a reducing agent; pyruvate; a glucocorticoid (when the cells to be cultured are hematopoietic cells); nucleosides for synthesis of DNA and RNA; growth factors that stimulate the proliferation and development of stromal cells and cells from a variety of tissues or organs, such as epidermal growth factor, fibroblast growth factor, platelet derived growth factor, and insulin; and extracellular matrix materials, such as collagen, fibronectin, and laminin.

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MEDIUM FOR LONG-TERM PROLIFERATION
AND DEVELOPMENT OF CELLS

BACKGROUND OF THE INVENTION

5

I. Field of the Invention

        This invention relates to serum-free or serum-
depleted culture media for supporting the proliferation
and development of cells. More particularly, it is

10   directed to culture media for the long-term growth of
hematopoietic cells and the stromal cells which support
their growth. It is also directed to methods for
culturing mammalian cells from different tissues and

15   organs using such media. The media and methods enable
the maintenance of cell growth and development for up to
several months.

II. Description of Background and Related Art

20

        One aspect of the present invention is a
medium for culturing cells, particularly mammalian cells
in the absence of or substantial depletion of serum,
whereby the medium is chemically well defined, while

25   still providing long-term support of development and
proliferation of such cells.

        In the past, many types of mammalian cells
have been isolated and attempts have been made to
propagate the cells in culture for further study or use.

30   The media used for such culture have typically included
sources of nutrients, adhesion factors and growth
factors required for cell proliferation and development,
many provided by horse, bovine or calf serum. However,
the use of serum to support cell culture has been

35   problematic since it does not provide physiological
conditions. Indeed, the only time a cell would see the

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sort of serum concentration normally used for cell culture
culture would be at the site of a wound, where the blood
is clotting. The use of serum is further complicated by
high cost and the presence of undefined components in
5   the serum which may vary from sample to sample. The
presence or absence of these undefined components has
led to inconsistent results in the culture of cells and
lack of control over the culturing process. For
example, a particular serum sample may introduce
10   particular components into the culture medium that
inhibit the growth of the cells.

         In response to problems associated with the
presence of serum, a variety of serum-free media have
been produced in the past. See for example:
15

         1)   Barnes, D. & Sato, G. Serum free cell
culture. A unifying approach. Cell. 1980; 22: 649;

         2)   Cormier, F., Ponting, I.L.O., Heyworth,
20   C.M. & Dexter, T.M. Serum-free culture of enriched
murine haemopoietic stem cells I: Effect of haemopoietic
growth factors on proliferation. Growth Factors.
1991; 4: 157-164;

25   3)   Deslex, S., Negrel, R. & Ailhaud, G.
Development of a chemically defined serum-free medium
for differentiation of rat adipose precursor cells.
Experimental Cell Research. 1987; 168: 15-30;

30   4)   Drouet, X., Douay, L., Giarratana, M.C.,
Baillou, C.L. Gorin, N.C., Salmon, C.H. & Najman, A.
Human long-term bone marrow culture in serum-free
medium. British Journal of Haematology. 1989; 73: 143-
147;
35   5)   Kumar, R.K., O'Grady, R., Li, W., Smith,
L.W., Rhodes, G.C. Primary culture of adult mouse lung

- 3 -

fibroblasts in serum-free medium: responses to growth
factors. Experimental Cell Research. 1991; 193: 398-
404;

5   6)   Migliaccio, G. Migliaccio, A.R. &
Adamson, J.W. The biology of hematopoietic growth
factors: Studies in vitro under serum-deprived
conditions. Experimental Hematology 1990; 18: 1049-
1055; and

10

             7)   Ponting, I.L.O., Heyworth, C.M., Cormier,
F. & Dexter, T.M. Serum-free culture of enriched murine
haemopoietic stem cells II: Effects of growth factors
and haemin on development. Growth Factors. 1991; 4: 165-
15 173.

             As described in a number of the cited
articles, there already exist methods for the short-term
(up to 3-4 weeks) serum-free culture of hematopoietic
20 progenitor cells, using added hematopoietic growth
factors as the proliferative and developmental stimuli.
However, the development of ideal serum-free media to
study the long-term culture of hematopoietic cells has
proven to be particularly difficult.
25   Prior to the invention described herein,
mammalian long-term hematopoiesis has been studied *in
vitro* mainly using serum supplemented bone marrow
culture systems which appear to closely mimic some of
the processes that occur *in vivo*. The first of these was
30 described by Dexter, T.M., *et al.*, J. Cell. Physiol.,
91: 335-344 (1977) and involves the formation of an
adherent layer of murine bone marrow stromal cells,
which included endothelial cells, fibroblasts,
adipocytes and macrophages. This cell layer was required
35 to support the hematopoietic cells, probably by
providing a physical adhesive matrix as well as the

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correct cell to cell signalling, including the necessary
growth factors for hematopoietic cell proliferation and
development.

     The hematopoiesis which takes place in these
5 Dexter cultures occurs in intimate contact with the
stromal cell layer. In many cases the stem cells and
progenitor cells proliferate under the stromal cells
forming foci called cobblestone regions due to their
characteristic appearance under phase microscopy. As the
10 hematopoietic cells mature, many of them migrate to the
uppermost surface of the stromal cells. Further
maturation to a fully differentiated state results in
release of the hematopoietic cells into the surrounding
medium where they are removed by the bi-weekly feeding.
15   This culture system enabled the maintenance of
hematopoiesis for several months, with the constant
production of progenitor cells. Soon after, a similar
system was developed for human cells (Gartner, S. and
Kaplan, H.S., Proc. Natl. Acad. Sci. USA, 77: 4756 -
20 4759 (1980)).

     One important drawback of the Dexter method
was that it mainly allowed the development of
macrophages and neutrophils, at the expense of other
cell types normally produced by the bone marrow,
25 particularly B lymphocytes. A method for the long-term
production of B lymphocytes was discovered by Whitlock,
C.A. and Witte, O.N., Proc. Natl. Acad. Sci. USA 79:
3608 - 3612 (1982). This system differed from that
described by Dexter, *et al.* in that the horse serum was
30 replaced with fetal calf serum and hydrocortisone was
replaced with 2-mercaptoethanol. However, this system
precluded the development of hematopoietic lineages
other than B lymphocytes. The reason(s) for the
differences in the development that occurs in these two
35 culture systems, and why they do not mimic all aspects

- 5 -

of bone marrow hematopoiesis, is unknown but is possibly a reflection of the use of serum.

In spite of the many attempts that have been made to produce an ideal, chemically controlled medium for culturing cells, especially mammalian hematopoietic cells, a number of shortcomings have persisted in such media. For example, prior media do not truly enable long-term culture of such cells. In accordance with the present invention, "long-term" is defined as greater than or equal to approximately eight weeks of continuous proliferation and development, including generation of new progenitor cells in the medium. The media of the present invention can produce up to three to five months of continuous growth and development in culture. There have been no prior serum-free media that have been able to achieve such long-term culture of mammalian hematopoietic cells.

The following is a brief description of some of the additional related prior art:

Cormier, et al. Growth Factors. 1991; 4: 157-164, relates to serum-free culture of enriched murine hematopoietic stem cells and focuses on the effect of certain growth factors on proliferation. However, the medium disclosed in this publication contains different components in different relative proportions as compared to the present invention, and was unable to achieve any long-term growth (as defined herein), in contrast to the present media.

Dexter, T.M., et al., J. Cell. Physiol. 91: 335-344 (1977), discloses the classical Dexter long-term bone marrow culture method. It is notable that this method contains serum, unlike the present invention. As a result its use is restricted, as already described, to

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certain mouse strains and even with such strains the
growth is not as pronounced as in the current invention.

Dexter, T.M., et al., Long-Term Bone Marrow
5   Culture. New York: Alan R. Liss: 57-96, is a review of
long-term marrow culture techniques and media. None of
the techniques or media disclosed in this reference
contain the same group of components as the present
invention.
10

Drouet, X., et al. Brit. J. of Haem. 73: 143-
147 (1989), disclose a human long-term bone marrow
culture medium that is serum-free. However, the medium
disclosed therein is not capable of achieving the same
15   degree of long-term culture as in the present invention,
growth being maintained for only 3-4 weeks. Furthermore,
unlike the media of the present invention, those
discussed by Drouet, et al. do not involve stromal
cells.
20

Whitlock, C.A., & Witte, O.N. P.N.A.S. 79:
3608-3612 (1982), relates to long-term culture of B
lymphocytes and their precursors from murine bone
marrow. This is the famous Witte-Whitlock culture
25   technique. However, again, the media disclosed in this
paper are substantially different from those claimed in
the present invention, in that they contain serum and
can only support B lymphocyte growth.

30   Teofili, L., et al. Ann. Hematol. 65: 22-25
(1992), describes a serum-free culture system for the
growth of human hematopoietic progenitor cells using a
preformed extracellular matrix. However, this method
does not produce long-term growth, progenitor cells only
35   being maintained in reasonable numbers for 3 weeks. In
addition, this method does not use growth factors to

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support the growth of the bone marrow stromal cells and
does not use purified extracellular matrix materials for
cell adherence. As a result it does not support the
growth of cells from a wide variety of tissues and
5   organs.

In spite of the above prior art, there remains
a need to produce chemically defined media for long-term
culture of cells. Ideally, such media should be simple
10   to prepare, contain chemically defined ingredients, be
economical to produce, and achieve optimal long-term
growth and development of cells.
Accordingly, it is an object of the present
invention to provide serum-free media for long-term
15   maintenance of proliferation and development of cells,
especially of hematopoietic origin.
A further object of the present invention is
to provide methods for both short and long-term culture
of cells in a chemically defined medium.
20   Another object of this invention is to provide
a method for stimulating the proliferation and/or
development of early progenitor cells for bone marrow
transplants and/or gene transfer into these cells for
gene therapy procedures.
25   These and other objects of the invention as
will hereinafter be described in greater detail will be
apparent to one of ordinary skill in the art.

30

III. Summary of the Invention

In accordance with the objects of the present
invention, there is provided a medium for long-term
35   proliferation and development of cells, which comprises
cell growth and development-effective amounts of:

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(a)   a standard culture medium such as
Iscove's modified Dulbecco's medium (IMDM), RPMI, DMEM,
Fischer's, alpha medium, Leibovitz's L-15, NCTC, F-10,
5   MEM and McCoy's;

(b)   serum albumin;

(c)   transferrin;

10

(d)   a source of lipids and fatty acids;

(e)   cholesterol;

15   (f)   a reducing agent;

(g)   pyruvate;

(h)   nucleosides for synthesis of DNA and RNA;
20

(i)   one or more growth factors that stimulate
the proliferation and development of stromal cells
and/or cells from a variety of organs and tissues
(preferable mammalian), such as epidermal growth factor,
25   basic fibroblast growth factor, platelet derived growth
factor, and insulin; and

(j)   one or more extracellular matrix
materials;
30

wherein said medium is serum-free or serum-depleted.

An optional component of the serum-free medium
which is used in cultures where neutrophil development
35   and proliferation is desired (e.g., long-term bone
marrow cultures) is:

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(k) a glucocorticoid such as hydrocortisone, cortisol, dexamethasone or other structurally related, natural or synthetic molecule.

Another optional component of the serum-free or serum-depleted medium which may be added separately to the medium when the cells to be supported are hematopoietic cells is:

(l) stromal cells, which provide a supporting network for hematopoietic cells, as described below in greater detail.

In an exemplary embodiment of the present invention, particularly suited for growth of neutrophils, the cell culture medium comprises the following components in the indicated concentrations:

Component	Suitable Conc.
Iscove's Modified Dulbecco's Medium	0.7 - 1.17 x
Bovine serum albumin	>1 mg/ml
Bovine transferrin	> 25 µg/ml
Soybean lipids	>5 µg/ml
Cholesterol	>1 µg/ml
2-mercaptoethanol	10 - 400 µM
Sodium pyruvate	>20 µg/ml
Hydrocortisone	0.05 - 5 µM
Nucleosides	1 - 100 µg/ml
Epidermal growth factor	> 0.5 ng/ml
Fibroblast growth factor	> 0.1 ng/ml

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Component	Suitable Conc.
Platelet-derived growth factor	> 0.5 ng/ml
Insulin	> 0.5 µg/ml
Collagen IV	> 1 µg/cm^2
Fibronectin	> 0.2 µg/cm^2

In a preferred embodiment of the present invention, the cell culture medium used to grow cells from different organs or tissues is as already described except that it does not contain any hydrocortisone, or other related glucocorticoid.

In another preferred embodiment, hydrocortisone or another glucocorticoid is not present in the medium and stimulation of the hematopoietic progenitor cells by the growth factor CSF-1, a developmental and proliferative stimulus for macrophages, is also prevented. This can be achieved in a number of ways. A mutant strain of mice can be used in which the CSF-1 gene (B6C3Fe-a/a-op/op mouse strain) or the gene for its receptor or part of the CSF-1 receptor signalling pathway is inactivated, so that no functional protein is produced. A variety of inhibitors of CSF-1 activity could also be employed. For example, neutralizing antibodies against CSF-1 or its receptor could be used to prevent stimulation. In addition it is possible to use antisense oligomers (short sequences of single stranded nucleic acids) to prevent the expression of CSF-1, its receptor or some part of the signal transduction mechanism. The hematopoietic development in these cultures is not restricted to neutrophils and macrophages, but also includes other cell types such as lymphoid, mast cell, megakaryocyte and erythroid cells.

In a particularly preferred embodiment, the cells to be cultured are mammalian in origin.

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In a further particularly preferred
embodiment, the cells are hematopoietic or lymphopoietic
in origin.

5   IV.   Brief Description of the Drawings

FIGURE 1 depicts a comparison of the current
invention (LTBMC-SFM) and prior serum-free media, in
terms of the number of bone marrow adherent cells
10   (stromal and hematopoietic cells) after 7 days. Note
that the present media gave very good hematopoiesis
after 2 weeks, whereas hematopoiesis was not observed in
any of the prior media tested.

FIGURE 2 depicts the effect of removing one of
15   the groups of serum-free medium components on the
establishment of long-term bone marrow cultures as
indicated by the adherent cell counts after 7 days.
Error bars show standard deviations.

FIGURE 3 depicts the effect of adding serum-
20   free medium components to serum supplemented "Dexter"
cultures (DEX), including growth factors (+ GFs),
Iscove's medium (+ IMDM) instead of Fischer's medium,
extracellular matrix materials (ECM), additional
nutrients (NUTS), and a temperature of 37°C. rather than
25   33°C. The cultures SFM + 37°C + DEX were set up under
standard serum-free medium conditions, but with horse
serum. Error bars show standard deviations.

FIGURE 4 depicts the relative number of
adherent cells (a combination of bone marrow stromal and
30   hematopoietic cells) in serum supplemented and
serum-free media after 7 days of culture. The importance
of extracellular matrix materials such as collagen and
fibronectin in serum-free cultures is also shown.
Fibronectin is at a conc. of 2 μg/cm^2. Error bars show
35   standard deviations.

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FIGURE 5 depicts the long-term hematopoiesis which occurs using the current invention, as indicated by the total number of non - adherent hematopoietic cells (mainly mature cells) produced over an 11 week
5   period, ± standard deviation.

FIGURE 6 depicts the long-term hematopoiesis which occurs using the current invention, as indicated by the number of non-adherent hematopoietic progenitor cells (colony forming cells) produced over a 10 week
10 period, ± standard deviation.

FIGURE 7 depicts the production of non-adherent hematopoietic cells after 21 days from serum-free long-term bone marrow cultures when using different strains of mice. C3H, C57Bl/6, Balb/c, and DBA/2 are
15 inbred strains of mice; CD-1 is an outbred strain of mouse; while B6D2F$_1$ (BDF1) and B6C3F$_1$ are hybrids (female C57Bl/6 x male DBA/2 and female C57Bl/6 x male C3H respectively). Error bars show standard errors.

FIGURE 8 shows a table which depicts the
20 morphology of the non-adherent hematopoietic cells produced in long-term bone marrow cultures of normal (+/+) mutant (op/op) mice in the presence or absence of hydrocortisone (HC).

25 V.   Detailed Description of the Invention

A.   Comparison of the Invention with Prior Media

The essential reason why prior serum-free
30 media have failed to give long-term growth of normal hematopoietic cells is that they have not contained the correct combination of ingredients in the proper concentration ranges to support the growth of a wide range of cell types, including hematopoietic cells and
35 stromal cells. This has resulted in many prior serum-free media being limited to supporting the growth of

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cell lines, rather than the more difficult to maintain,
normal primary cells (i.e., those cells that come
directly from an animal). Those media that are capable
of maintaining the growth of normal cells only do so for
5   one or a few cell types, and do not provide permissive
growth conditions for the long-term culture of
hematopoietic cells and stromal cells. In contrast,
media of the present invention have the unique ability
to support the growth of very different cells from a
10   wide variety of tissues and organs, including primary
(normal) cells and cell lines. This ability enables the
long-term growth of both the hematopoietic progenitor
cells as well as the bone marrow stromal cells
(adipocytes, macrophages, endothelial cells and
15   fibroblasts) which are essential for supporting the
growth of the hematopoietic cells. This is an ability
that prior serum-free media do not possess.

        Another shortcoming of prior serum-free media
was their species specificity; in contrast, the media of
20   the current invention can support the growth of both
mouse and human cells, and they are expected to support
the growth and development of cells from other species
as well.

        While the current invention is a dramatic
25   improvement over prior serum-free media, it is also an
improvement over standard serum-supplemented bone marrow
cultures. For example, present techniques are limited in
the strains of mice from which long-term hematopoiesis
can be obtained, whereas no such limitation exists for
30   the current invention, hematopoiesis being obtained from
every strain tested. Furthermore, the rate of bone
marrow stromal and hematopoietic cell growth under the
conditions described in the present invention greatly
exceed those in serum-supplemented cultures due to the
35   improvement of numerous aspects of the culture
conditions, e.g. adherence, temperature, nutrients and

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growth factors. In this way, the 4-5 week long period
required for classical serum- supplemented long-term
bone marrow cultures to produce significant numbers of
hematopoietic cells can be reduced to just around 2

5 weeks.

        A further major advantage of the media of this
invention over prior methods for the long-term culture
of hematopoietic progenitor cells which use serum is the
control which it allows in manipulating the

10 proliferation and development of the hematopoietic
cells. The reason for this is that all of the
ingredients and their concentrations in the culture
system are known and can therefore be controlled. This
contrasts to serum supplemented cultures where the

15 contents of the serum are generally unknown, and where
the concentrations of the known components are similarly
undefined.

        For example, by decreasing the concentration
of one of the culture ingredients it is possible to

20 efficiently make the culture conditions sub-optimal for
growth, thus reducing the number of hematopoietic and/or
stromal cells produced.

        In a similar way changes in hematopoietic stem
cell development can be induced by the removal of the

25 serum-free medium ingredients, hydrocortisone, which is
required for optimal growth of neutrophilic cells; as
well as the removal of the growth factor CSF-1, a
stimulus for macrophage growth, normally produced by the
stromal cells in the culture. These alterations in the

30 culture result in not only neutrophil and macrophage
development occurring, but also lymphoid, mast cell and
possibly erythroid and megakaryocyte development as
well. This type of varied development is far more
representative of that which occurs in the bone marrow

35 *in vivo*, and is not observed in any of the prior

- 15 -

techniques for the long-term culture of hematopoietic
cells.

The use of a defined culture system also makes
possible the precise determination of the effect of a
5   known molecule on the cells. Such an analysis is not
possible using serum-supplemented cultures where the
test molecule may already be present at an unknown
concentration.

10   B.   Definitions

In general the "medium" and "media" as used in
connection with the present invention are solutions
containing growth factors and nutrients, which are used
15   to support the growth and development of cells.

By "long-term" is meant continuous growth and
development of the cells being cultured, as well as
generation of progenitor cells, for a time period of at
least about 8 weeks and up to or surpassing about 12 -
20   20 weeks. Preferably, "long-term" means 8-12 weeks.

The "cells" that may be supported (i.e., grown
and developed/differentiated) by the media of the
present invention are preferably mammalian cells, such
as: human, monkey, bovine, ovine, equine, and murine.
25   They are preferably, human or murine cells. Cells from
other sources as well as from other mammalian species
are also contemplated as would be apparent to one of
ordinary skill in the art.

Such cells may be derived from a variety of
30   "tissues," such as lung, liver, kidney, thymus, thyroid,
heart, brain, and the like.

Particular examples of normal mammalian cells
that may be cultured in the media of the present
invention are fibroblasts, endothelial cells,
35   adipocytes, glial cells, neuronal cells, myoblasts,
epithelial cells (which may be from a wide range of

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different tissues), hepatocytes, osteoclasts, and heart
muscle cells. The culture media also support a wide
range of cell lines including immortalized versions of
those already listed; examples of others include bone

5   marrow stromal cell lines, embryonic stem cell lines
(such as: D3, E3, SQ1.2S8, MBL-1, 632, and CCEG2),
embryonic carcinoma cell lines (such as PGC3), melanoma,
mammary, and pituitary cell lines.

Preferred cells of the present invention to be

10 cultured are hematopoietic and lymphopoietic cells which
are preferably obtained from the spleen, fetal liver,
peripheral blood, umbilical cord or bone marrow.

In general, the present culture media have
successfully supported the growth of every cell type so

15 far tested and it is anticipated that it will support
the growth of many mammalian cell types.

The term "stromal cells" is used to refer to
those cells of the bone marrow which form a supporting
network for hematopoietic cells *in vivo* and *in vitro*. In

20 this respect they support the hematopoietic cells both
physically in providing sites of attachment, as well as
biologically in providing the cytokines which these
cells require for their growth. In culture they form an
adherent layer and include endothelial cells,

25 macrophages, fibroblasts and pre-adipocytes/adipocytes.
Accordingly, when the cells to be supported are
hematopoietic cells, stromal cells will preferably also
be included either as part of the medium, or added to
the medium (preferably, $10^4 - 10^7$ stromal cells per

30 culture) during the time the hematopoietic cells are
present in the medium.

The term "amino acid" refers to all naturally
occurring alpha amino acids in both their D and L
stereoisomeric forms, and their analogs and derivatives.

35 An analog is defined as a substitution of an atom in the
amino acid with a different atom that usually has

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similar properties. A derivative is defined as an amino
acid that has another molecule or atom attached to it.
Derivatives would include, for example, acetylation of
an amino group, amination of a carboxyl group, or
5   oxidation of the sulfur residues of two cysteine
molecules to form cystine.

        The amino acids are identified by either the
standard single-letter or three-letter designations.

        "Serum-free" is used herein to mean that
10   substantially all serum is excluded from the medium (for
example 0-0.49 %). Further to this is the term "serum-
depleted" which means that up to about 5% of serum could
be added to the medium (for example 0.5-5 %).
Preferably less than 3% of the medium will be derived
15   from serum, particularly preferably less than 0.5%. In
contrast, serum-containing media of the prior art have
typically included >5-40% serum.

        By "cell culture effective amount" is meant an
amount that is capable of producing long-term cell
20   proliferation and development. Such amounts may be
determined for a given constituent by experimentation
wherein varying concentrations of a given constituent
are added to cell growth media, and the extent and
duration of the cell proliferation and development is
25   determined by methods known to a skilled cell culturist.
Preferred ranges and examples of such amounts are
provided throughout the specification.

        C.   Description of Culture Media

30

        An important aspect of the present invention
is the discovery that combinations of ingredients in the
proper amounts can support both short- and long-term
growth (i.e. proliferation) and development of mammalian
35   cells, particularly hematopoietic cells.

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In a general sense, there are three primary types of materials included in the present media:

Extracellular matrix materials

These are involved in adhesion of cells, such
5   as to the culture surface. They are typically included in a concentration of 0.2 - 100 $\mu g/cm^2$.

Examples of extracellular matrix materials from mammalian sources (bovine or human, though preferably murine) that are preferred are: a source of
10 collagen (preferably collagen I and collagen IV), fibronectin, vitronectin and laminin. Especially preferred are:

•   Mouse collagen IV, which can be used at a concentration of greater than 1 $\mu g/cm^2$, preferably from
15 about 2 - 100 $\mu g/cm^2$, and more preferably at about 5$\mu g/cm^2$.

•   Mouse fibronectin, which can be used at a concentration of greater than 0.2 $\mu g/cm^2$, preferably from about 0.5 - 100 $\mu g/cm^2$, and more preferably at
20 about 2 $\mu g/cm^2$.

However, less pure sources of extracellular matrix can also be used, such as MATRIGEL, which is a complex mixture of matrix and associated molecules. Matrix secreted by a previously adherent cell can also
25 be used. The cell is grown on the plastic culture surface and secretes extracellular matrix, removal of these cells then provides a layer of extracellular matrix to which a second cell type can adhere.

30 Growth factors

These are molecules involved in stimulating proliferation of the stromal cells or other cells from a variety of tissues that are desired to be supported by the media. In the case of bone marrow stromal cells they
35 produce growth factors for the hematopoietic cells in the culture, e.g. GM-CSF, G-CSF, SCF, IL-1, CSF-1, IL-6,

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and a variety of others. In cases where a purified
population of hematopoietic cells is cultured using the
serum-free medium these growth factors have to be added
(typically in concentrations of 0.5 ng/ml - 100 µg/ml,
5   depending on the growth factor and the cells involved),
since the stromal cells will have been removed.

The choice of growth factors required to
support the growth and development of particular types
of cells will vary depending on the cell type, and will
10   be readily determinable by those skilled in the art of
cell biology and culture. Growth factors for the stromal
and tissue cells that are particularly useful in
accordance with the present invention, and to which
additional specific growth factors can always be added
15   as desired or necessary for growth include:

•   Recombinant human epidermal growth factor
(EGF), which can be used at a concentration of greater
than 0.5 ng/ml, preferably from about 5 - 200 ng/ml, and
more preferably at about 15 ng/ml.

20   •   Recombinant human basic fibroblast growth
factor (bFGF), which can be used at a concentration of
greater than 0.1 ng/ml, preferably from about 0.5 - 40
ng/ml, and more preferably at about 2 ng/ml.

•   Recombinant human platelet derived growth
25   factor (PDGF) (preferably the B-B isoform), which can be
used at a concentration of greater than 0.5 ng/ml,
preferably from about 2 - 200 ng/ml, and more preferably
at about 10 ng/ml.

•   Insulin (human or preferably from bovine
30   pancreas) can be used at a concentration of greater than
0.5 µg/ml, preferably from about 2 - 100 µg/ml, and more
preferably at about 10 µg/ml.

Less pure sources of growth factors such as
conditioned media from cell lines producing these growth
35   factors can also be used, but do not give such a defined

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culture. In addition it is possible to use growth
factors from other species, e.g. bovine and mouse.

Nutrients

5   These are molecules involved in normal
metabolic functions of the cultured cells.

          Nutrients and metabolic additives useful in
the media of the present invention include a standard
culture medium (described below); serum albumin

10   (preferably bovine or human); transferrin (preferably
bovine or human); a source of lipids and fatty acids
useful in cell growth and development (preferably
soybean lipids); cholesterol; a reducing agent
(preferably 2-mercaptoethanol or monothioglycerol);

15   pyruvate (preferably sodium pyruvate); a glucocorticoid
(preferably, a cortisone derivative such as
hydrocortisone 21-hemisuccinate, particularly preferably
the sodium salt); and nucleosides.

          The following is a more detailed description

20   of some of the individual components of the nutrients in
the media of the present invention:

          •   The "standard culture media" which may be
employed in accordance with the present invention are
standard culture media for growing cells that typically

25   provide at least one component from the following
categories:

          (a)   an energy source, usually in the
form of a carbohydrate such as glucose;

          (b)   substantially all essential and non-

30   essential amino acids, usually the basic set of twenty
amino acids, plus cystine instead of cysteine;

          (c)   vitamins and/or other cell-growth
supporting organic compounds required at low
concentrations;

35   (d)   a buffering agent such as HEPES,
Tris, or MOPS (preferably HEPES), which act to stabilize

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the hydrogen ion concentration and therefore the pH of
the solution by neutralizing, within limits, both acids
and bases;

(e) inorganic salts and trace elements,
5   where trace elements are defined as inorganic compounds
or naturally occurring elements that are typically
required at very low concentrations, usually in the
micromolar range (e.g., 1 - 1000 μM).

Particular examples of the standard culture
10 media which are useful in accordance with the present
invention are: RPMI, DMEM, MEM, Leibovitz's L-15,
Fischer's, F-10, alpha medium, NCTC and McCoy's, but
preferably Iscove's modified Dulbecco's medium (IMDM);
Iscove, N.N. & Melchers, F. J. Exp. Med. 147, 923
15 (1978).

In accordance with this invention, the
standard culture medium is supplemented with particular
components from the following categories:

The "serum albumin" of the present invention
20 may be obtained from any mammalian source, such as
human, monkey, bovine, ovine, equine, murine. Preferably
the source of serum albumin is human or bovine. The
serum albumin can be used at a concentration of greater
than 1 mg/ml, preferably from about 3 - 50 mg/ml, and
25 more preferably at about 10 mg/ml. It is notable that
although the present media are generally serum-free,
certain serum components which are chemically well
defined and highly purified (>95% pure), such as serum
albumin, may be included.

30   Transferrin is another protein component of
the media set forth herein which is also preferably of
mammalian origin, particularly preferably human or
bovine. This component can be incorporated at greater
than 25 μg/ml, preferably from about 25 - 1000 μg/ml,
35 and more preferably at about 100 - 300 μg/ml.

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"A source of lipids and fatty acids" means any
of a variety of such sources. For example, crude
extracts of lipids may be used to supplement the media
of this invention. The lipid extract may be obtained
5   from any biological source, although one preferred
source is plant material, and particularly soybeans
(soybean lipid extract may be purchased from Boehringer-
Mannheim). Typically, the extract will contain from
about twenty percent to about ninety-five percent lipid,
10   the majority of which will be phospholipid, the
remainder being fatty acids, with traces of sterol. The
extract may be added to the cell culture medium at a
concentration of greater than about 5 μg per ml,
preferably from about 5 μg per ml to about 100 μg per
15   ml, and more preferably 5 μg per ml to about 50 μg per
ml.

The addition of a molecule capable of
regulating the cell plasma-membrane fluidity is an
important component. Preferably, this invention employs
20   the lipid cholesterol at a concentration of greater than
about 1 μg/ml, or preferably from 3 - 30 μg/ml, or more
preferably at about 7.8 μg/ml.

The term "a reducing agent" means any reducing
agent that is compatible with a cell culture medium.
25   Preferred examples are: 2-mercaptoethanol or
monothioglycerol. Generally, the reducing agent is
present in a concentration of about 10 to 400 μM,
preferably 30 to 300 μM, and particularly preferably,
about 100 μM.

30   "Pyruvate" refers to any cell-culture
compatible salt of pyruvic acid, preferable sodium or
potassium pyruvate. Pyrucic acid itself may also be
added, forming a salt to some degree in situ. Pyruvate
is required for general metabolic processes as a
35   potential source of energy and for synthesizing more
complex molecules. Generally, the pyruvate is present

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in a concentration of 20 µg/ml, preferably 30 to 500
µg/ml, and particularly preferably, about 110 µg/ml.

The term "nucleosides" refers to naturally
occurring nucleosides that form the basis for synthesis

5   of DNA and RNA. Such nucleosides include the following:
adenosine, guanosine, cytidine, uridine, 2'-
deoxyadenosine, 2'-deoxyguanosine, 2'-deoxycytidine and
thymidine. Phosphorylated (mono, di, or tri) analogues
could also be used. The nucleosides may be added to the

10   cell culture medium at a concentration of about 1 to 100
µg/ml, preferably from about 5 to 30 µg/ml and
particularly preferably about 10 µg/ml.

The growth factors epidermal growth factor,
fibroblast growth factor, platelet-derived growth factor

15   and/or insulin which have already been discussed in
detail in section B are prferably all included in the
standard medium.

A component which is not required in all
embodiments of the invention is a natural or synthetic

20   corticoid such as the cortisone derivatives: prednisone,
dexamethasone or preferably hydrocortisone. These
molecules are involved in carbohydrate, protein and fat
metabolism, are anti-inflammatory and immunosuppressive,
and are thought to regulate the levels of certain

25   cytokines and/or their receptors, such as TNF-alpha,
GM-CSF and G-CSF. A corticoid such as hydrocortisone can
be used at a concentration of about 0.05 - 5 µM, or
preferably 0.3 - 1 µM, or particularly preferably at
about 0.5 µM.

30   The following table provides a summary list of
some particularly preferred components along with
suitable concentrations, preferred concentrations and
the most preferred concentrations for each component.
These quantities are applicable to equivalent components

35   of each type defined herein listed in the table.

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Table of Culture Components and Concentrations

Component	Suitable Exemplary Conc.	Preferred Conc.	Conc.
Standard Culture Medium (e.g., Iscove's Modified Dulbecco's Medium)	0.7 - 1.17 x	0.8 - 1.09 x	1 x
Serum albumin (e.g., bovine)	> 1 mg/ml	3 - 50 mg/ml	10 mg/ml
Transferrin (e.g., bovine)	> 25 µg/ml	25 - 1000 µg/ml	100 µg/ml
Source of lipids (e.g., soybean lipids)	> 5 µg/ml	5 - 100 µg/ml	25 µg/ml
Cholesterol	> 1 µg/ml	3 - 30 µg/ml	7 .8 µg/ml
Reducing Agent (e.g., 2-mercaptoethanol)	10 - 400 µM.	30 - 300 µM.	100 µM
Pyruvate (e.g., sodium pyruvate)	> 20 µg/ml	30 - 500 µg/ml	110 µg/ml
Glucocorticoid (e.g., Hydrocortisone)	0.05 - 5 µM.	0.3 - 1 µM.	0.5 µM
Nucleosides (e.g.,DNA/RNA derived)	1 - 100 µg/ml	5 - 30 µg/ml	10 µg/ml
Growth Factors			
(e.g., Epidermal growth factor	> 0.5 ng/ml	5 - 200 ng/ml	15 ng/ml
Fibroblast growth factor	> 0.1 ng/ml	0.5 - 40 ng/ml	2 ng/ml
Platelet-derived growth factor	> 0.5 ng/ml	2 - 200 ng/ml	10 ng/ml
Insulin)	> 0.5 µg/ml	2 - 100 µg/ml	10 µg/ml

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(Table of Culture Components and Concentrations
Continued)

5   Extracellular
    Matrix Materials

    (e.g.,
    Collagen IV   > 1 µg/cm^2 2 - 100 µg/cm^2 5 µg/cm^2
10
    Fibronectin)   > 0.2 µg/cm^2 0.5 - 100 µg/cm^2 2 µg/cm^2

        One specific, exemplary cell culture medium
15 within the scope of the present invention is set forth

    as follows:

    Component   **Concentration (mg/L)**
                                           (unless stated otherwise)
20

    ISCOVE'S MODIFIED DULBECCO'S MEDIUM

    INORGANIC SALTS:
        $CaCl_2$ (anhydr.)   165.00
25   KCl   330.00
        KNO_3 0.076
        $MgSO_4$ (anhydr.)   97.67
        NaCl   4505.00
        $NaHCO_3$ 3024.00
30   NaH_2PO_4 . H_2O 125.00
        Na_2SeO_3 . 5 H_2O 0.0173

    OTHER COMPONENTS:
        D-Glucose   4500.00
35   Phenol red   15.00
        HEPES   5958.00
        Sodium pyruvate   110.00

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AMINO ACIDS:

	L-Alanine	25.00
	L-Asparagine . H_2O	28.40
	L-Arginine . HCl	84.00
5	L-Aspartic acid	30.00
	L-Cystine . 2HCl	91.24
	L-Glutamic acid	75.00
	L-Glutamine	584.00
	Glycine	30.00
10	L-Histidine HCl . H_2O	42.00
	L-Isoleucine	105.00
	L-Leucine	105.00
	L-Lysine HCl	146.00
	L-Methionine	30.00
15	L-Phenylalanine	66.00
	L-Proline	40.00
	L-Serine	42.00
	L-Threonine	95.00
	L-Tryptophane	16.00
20	L-Tyrosine (Disodium salt)	104.2
	L-Valine	94.00

VITAMINS:

	Biotin	0.013
25	Ca D-Pantothenate	4.00
	Choline chloride	4.00
	Folic acid	4.00
	i-inositol	7.20
	Nicotinamide	4.00
30	Pyridoxal HCl	4.00
	Riboflavin	0.40
	Thiamine HCl	4.00
	Vitamin B_{12}	0.013

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ADDITIONAL COMPONENTS

	Bovine Serum Albumin	10000.00
	Bovine Transferrin	100.00
	Soybean Lipids	25.00
5	Cholesterol	7.80
	Hydrocortisone	0.24
	2-mercaptoethanol	7.84
	Sodium Pyruvate	110.00
	Adenosine	10.00
10	Cytidine	10.00
	Guanosine	10.00
	Uridine	10.00
	2'-deoxyadenosine	10.00
	2'-deoxyguanosine	10.00
15	2'-deoxycytidine	10.00
	Thymidine	10.00
	Epidermal growth factor	15×10^{-3}
	Fibroblast growth factor	2×10^{-3}
	Platelet-derived growth factor	10×10^{-3}
20	Insulin	10.00
	Collagen IV	$5 \ \mu g/cm^2$
	Fibronectin	$2 \ \mu g/cm^2$

D. Preparation of the Media and Culture

25 Technique

The following is an exemplary method for preparing the media and carrying out the culture technique of the present invention. Equivalent

30 components as set forth herein could be substituted within the concentration ranges set forth herein for those described below.

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Extracellular matrix (ECM)

The evening before initiating the cultures,
six well plates (Corning, 10 cm^2 surface area/well) are
5   coated with 0.73 ml solution of Iscove's Modified
Dulbecco's Medium (IMDM) containing glutamine (292
µg/ml), penicillin G (100 units/ml) and streptomycin
sulfate (100 µg/ml), all three are supplied together
(Irvine Scientific) at a 100x concentration. Dissolved
10   extracellular matrix materials can then be added to this
solution. A number of ECM molecules have been tested.
It has been determined that a source of collagen is
important, mouse collagen IV (5 µg/cm^2 Sigma, Cat.
#C0543) being preferred. Additional ECM molecules can
15   then be added, e.g. fibronectin and laminin, mouse
fibronectin (2 µg/cm^2 Sigma, Cat. #F1141) in combination
with collagen IV is used normally, in a final volume of
0.8 ml.
The next morning the wells are washed once
20   with IMDM and the serum-free medium and cells added.

Nutrient/Metabolic Additives

A number of nutrients are added to the medium,
25   they are prepared at a 100x final concentration, the
final concentrations in the culture are shown below:

•   Bovine serum albumin (10 mg/ml, Sigma,
cat.# A8412).
30   •   Bovine (100 µg/ml, Sigma cat.# T-1283) or
human transferrin (300 µg/ml, Boehringer-
Mannheim cat.# 652 202) (for mouse and human
cultures, respectively).
•   Soybean lipids (25 µg/ml, Boehringer-
35   Mannheim cat.#652 229).